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Short communication

Influence of yeast extract enrichment and *Pycnoporus sanguineus* inoculum on the dephenolisation of sugar-cane bagasse for production of second-generation ethanol

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ABSTRACT

Pretreatment of sugar-cane bagasse (SCB) by laccases from *Pycnoporus sanguineus* under solid-state fermentation (SSF) can favour dephenolisation of the substrate prior to ethanol production. To define adequate conditions for this biological pretreatment, an experimental design and surface plot analysis were performed using as independent variables: yeast extract, fungal inoculum and substrate amounts after different incubation times. Laccase and cellulase activities and chemical characterisation of the substrate by ^{13}C CP/MAS (cross-polarization at magic angle spinning) NMR (nuclear magnetic resonance) were used as responses. After 60 days of SSF, yeast extract enrichment and low amounts of inoculum enhanced dephenolisation of SCB. Moreover, yeast extract reduced the quantity of crystalline cellulose without strongly promoting cellulases and polysaccharide degradation. High amounts of inoculum decreased laccase activities and substrate dephenolisation.

1. Introduction

Production of second-generation bioethanol is based on the valorisation of agricultural wastes, like sugarcane bagasse (SCB). For instance, 18 Mt of SCB were produced in 2018 in Mexico [1]. Since ethanol production yield can reach 0.25 g/g of dry biomass of SCB [2], production of second-generation ethanol based on SCB can be considered as a relevant and sustainable technology that could feed Mexican demand for this type of resource. However, lignocellulosic material requires a pretreatment i.e. dephenolisation, in order to make the cellulose fibres accessible for the next step of fermentation. Enzymatic pretreatment appears to be a suitable eco-friendly technology, not requiring chemical or physical treatments, using low-energy input and mild operating conditions [3].

Dephenolisation of the complex polyphenolic structure of lignin can be performed by white-rot fungus like *Pycnoporus sanguineus*, which produces mainly laccases as phenoloxidases [4]. Laccases are particularly interesting enzymes for industrial applications since they do not require any co-substrate (neither H_2O_2 nor Mn), and their activities remain stable for several days [5]. In general, production of laccases has been shown to depend on the nature of nitrogen sources as well as on C/N ratio [6]. Generally, inorganic nitrogen sources lead to low laccase activities, while organic nitrogen sources result in high laccase yields [7]. Moreover, it seems that laccase activities could increase by supplementation of an enriched organic nitrogen source [8]. There are several reports using yeast extract as an enrichment to enhance production of laccase during SSF. Niladevi et al. [9], studied laccase activities of *Streptomyces psammoticus*, and showed an increase in these enzyme activities of 86% using yeast extract compared to NH_4Cl and of 39% compared to tryptone. Also, Fenice et al. [10], increased up to 22% laccase activities and up to 15% biomass production of *Panus tigrinus* with an enrichment of 0.1% of yeast extract and 0.5% of saccharose. Moreover, enrichment with yeast extract can induce different isoforms of laccases and enhance laccase activities overall [11]. The time of incubation and the amount of initial inoculum can also influence the expression of laccases and consequently dephenolisation of the SCB. It has been observed that during the first steps of SSF, laccase activities increased [8], and aromatic degradation can be observed days after inoculation occurred [12].

This study aimed to define the conditions of substrate pretreatment

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which favoured dephenolisation of SCB. A response-surface experimental design was set up to test the effects of yeast extract, the amount of fungal inoculum and of substrate, on the kinetic of SCB dephenolisation by *Pycnoporus sanguineus*. As responses, laccase and cellulase activities were measured and transformation of lignocellulose was evaluated *via* solid-state ^{13}C CP/MAS NMR.

2. Materials and methods

2.1. Strains and inoculum

The strain of *Pycnoporus sanguineus* used was isolated from a wild mushroom found in the sugar-cane fields of Jalcomulco, Veracruz, Mexico (19° 20′00′′N, 96° 46′ 00 ′W), registered as F. Ramírez-Guillén 932 in the XAL herbarium (INECOL, A.C.) and identified by analysis of ITS (internal transcribed spacer) fragments 1 and 4 (Access Genebank KR013138). The fungal strain was reactivated in plates of malt extract agar 1.5% at 30 °C for seven days. Then, five agar pieces of 1 cm diameter were used as inoculum for mycelial colonisation of wheat grains (10 days at 30 °C). Wheat grains were prepared as follows: 100 g of wheat grains were complemented with 150 ml of water and 0.5 g of gypsum and then sterilized by autoclaving [13].

2.2. Mesocosm preparation

The substrate was composed of 90% of SCB complemented with 10% of coffee pulp used as an inductor of laccases [14,15], which was previously milled and sterilized twice by autoclave. Dried and milled SCB (particles from 0.5 to 3 cm) was received from Mahuixtlan sugar refinery, located at Mahuixtlan, Veracruz, Mexico. The composition of this by-product was analysed by ^{13}C CP/MAS NMR by Hernández et al. (2017): O-Alkyl 79.76%, aromatics 12.87%, COOH 3.57% and Alkyl 2.45%. The coffee pulp was obtained from a shade-grown commercial coffee culture with the dominant variety *Coffea arabica* [16], located in the Tlacontla farm, San Marcos de León, municipality of Xico, Veracruz, Mexico. Each mesocosm was composed of 70 g of substrate (dry weight, DW) in 0.5 L aluminium plates (10*14*5 cm^3), covered with an aluminium foil. For each treatment, three replicates were made, to obtain a total of 99 mesocosms and incubated at 30 °C at 80% of the water holding capacity (WHC) in the dark. 33 mesocosms were analysed after 20, 40 or 60 days of incubation.

2.3. Experimental design

Three-factor experimental design was performed using as independent variables: the amount of yeast extract, inoculum and substrate using 4 levels and a full-cubic model (Table 1). The concentrations of yeast extract ranged between 0.7–9.1% DW (from 0.7 to 9.1 g) and the concentrations of inoculum from 5.0 to 14.3% DW (from 5.0 to 14.3 g). The rest of the mesocosm was composed of the substrate to keep the mesocosm weight constant (85.6 – 94.3%). Sampling was performed at 3 incubation times (20, 40 and 60 days of incubation). The dependent variables measured were: laccase and cellulase activities, and chemical properties of the substrate by solid-state ^{13}C CP/MAS NMR.

Table 1
Factors and levels evaluated in the experimental design (yeast extract ranging from 0 0.7–9.1% DW and fungal inoculum from 5.0 to 14.3% DW and substrate from 85.6–94.3%).

Conditions	Inoculum (%)	Yeast extract (%)	Substrate (%)
1	8.1	6.3	85.1
2	8.1	3.5	88.4
3	5.0	9.1	85.9
4	14.3	0.7	85.0
5	5.0	6.3	88.7
6	8.1	0.7	91.2
7	5.0	3.5	91.5
8	5.0	0.7	94.3
9	8.1	3.5	88.4
10	11.2	0.7	88.1
11	11.2	3.5	85.3

2.4. Enzymatic activities

Laccases and cellulases were quantified after 20, 40 and 60 days of incubation. The reaction mixtures for laccase were performed according to Farnet et al. [17], with syringaldazine (15 μM in methanol) as substrate. Cellulase activity was assayed using CarboxyMethylCellulose (CMC) at 1% as substrate according to Somogyi Nelson method modified by Farnet et al. [18]. One unit (U) of enzyme activities is defined as one μmole of the product formed per min and per g of dry weight (DW).

2.5. Solid-state ^{13}C CP/MAS NMR

The chemical composition of SCB, coffee pulp and of each mesocosm was characterized by solid-state ^{13}C CP/MAS NMR, on a spectrophotometer Bruker DSX 400 MHz operating at 100.7 MHz. Samples (400 mg) were spun at 10 kHz at the magic angle. Contact times of 2 ms were applied with a pulse width of 2.8 μs and a recycle delay of 3 s. Chemical shift values were referenced to tetra-methyl-silane and calibrated to glycine carbonyl signal set at 176.03 ppm. The relative C distribution in ^{13}C NMR spectra was determined by integrating the signal intensity in different chemical shift regions [19], with an integration routine supplied with Dmfit 2003 software. Seven common chemical shift regions were defined according to Mathers and Xu [20]. A decomposition index (Alkyl-C/O-Alkyl-C) was calculated according to Baldock et al. [21], as the humification ratio and the crystallinity of cellulose was also measured using the relative percentage of crystalline cellulose peak (90 ppm) *vs* the sum of the relative percentages of amorphous (83 ppm) and crystalline cellulose [22].

2.6. Statistical analyses

Correlations between variables and the factors tested were determined using UNSCRAMBLER 10*3 (CAMO). To find out whether the independent factors had significant effects on each response, a three-way ANOVA (analysis of variance) was carried out. Homoscedasticity and normality were analysed previously. The interaction effects of yeast extract, inoculum and substrate amounts were analysed by response surface methodology [23]. Pearson's correlation coefficients were calculated between all the NMR peaks from each 33 mesocosms after 60 days of incubation and a principal-component analysis (PCA) was performed to describe chemical changes of the substrate depending on the conditions tested.

3. Results and discussion

3.1. Enhancing laccase activities and dephenolisation of SCB

According to the surface plots (Fig. 1A), laccase activities were favoured by higher amounts of yeast extract during fermentation (P = 0.001). This complementation rich in amino acids, vitamins, sugars and ions like Cu^{2+} could enhance fungal metabolism [24] and increase production of multicopper oxidases such as laccases [25]. Here, yeast extract was used to test the effect of substrate complementation by a complex source of nitrogen and growth factors and this study revealed that it can indeed enhanced laccase activities. Nevertheless, for an industrial approach, a sustainable and affordable complementation using agroindustrial by-products should be implemented. Zimbardi et al. [4], used wheat straw (a culture substrate